Reprinted from APPLIED MICROBIOLOGY
Vol. 8, No. 3, May 1960
Printed in U.S.A.

R59-4

Fungicide Assay by Spore Germination in Shaker Flasks

RICHARD T. DARBY

Chemicals and Plastics Division, Quartermaster Research and Engineering Command, Quartermaster Research and Engineering Center, U. S. Army, Natick, Massachusetts

Received for publication September 30, 1959

Since the introduction in 1933 (Kluyver and Perquin, 1933) of the shaker flask method of culturing fungi this technique has found widespread use in fungus physiology (Foster, 1949). It was used for spore germination studies by Davies *et al.* (1948) and Mandels and Norton (1948). The measurement of antifungal activity, however, has been accomplished by the more conventional methods making use of agar plates, hanging drops, moist chambers, and more elaborate techniques (Horsfall, 1946). This paper describes the assay of fungicides by spore germination in shaker flasks.

MATERIALS AND METHODS

The required number of shaker flasks (125-ml Erlenmeyer) were fastened to a shallow tray by means of double-coated pressure-sensitive tape.[1] The flasks were loaded using a 5-ml automatic pipette. The several components were made up in concentrated form so as to give the final desired concentration when mutually diluted with the others. Aseptic methods are not necessary. The usual order for loading the flasks was as follows: buffer, substrate, fungicide, spore suspension. All flasks were brought to the same final volume with distilled water. The loaded flasks were loosely plugged, and the tray was transferred to a shaking machine in an incubator. The tray was also attached to the shaker by double-coated tape. After the desired incubation (4 hr), the cultures were removed and either preserved with formalin for future study or sampled and mounted in lacto-phenol plus cotton blue. Since the process of germination in fungus spores is progressive over a period of time and blends gradually into hyphal growth, it is necessary to decide arbitrarily at what point a given spore may be considered germinated. This is here defined as the stage at which the protruding germ tube is as long as it is wide as illustrated by Mandels and Darby (1953). Per cent germination was calculated from a count of several hundred spores.

In experiments reported here the buffer used was 0.025 M potassium phosphate, pH 6.8; the substrate was 0.1 per cent sucrose plus 0.1 per cent yeast extract; the organism was *Myrothecium verrucaria* (Alb. and Schw.) Ditm. ex Fr. strain QM 460. The method of propagating the organism and preparing the spore suspension have been previously described (Mandels and Darby, 1953). The washed suspension was adjusted to provide a final concentration of 1 to 5 $\times$ 10^5 spores per ml. The flasks were shaken at 225 rpm on a rotary shaker at 30 C.

[1] The pressure-sensitive tape no. 400 (Minnesota Mining and Manufacturing Company, St. Paul, Minnesota) has been found suitable.

RESULTS

The method is illustrated by the results of three studies: an ordinary dosage-response test, a kinetic study, and a study of fungicides in combination.

For single compounds the results of three replicate experiments are shown in figure 1 in which sodium

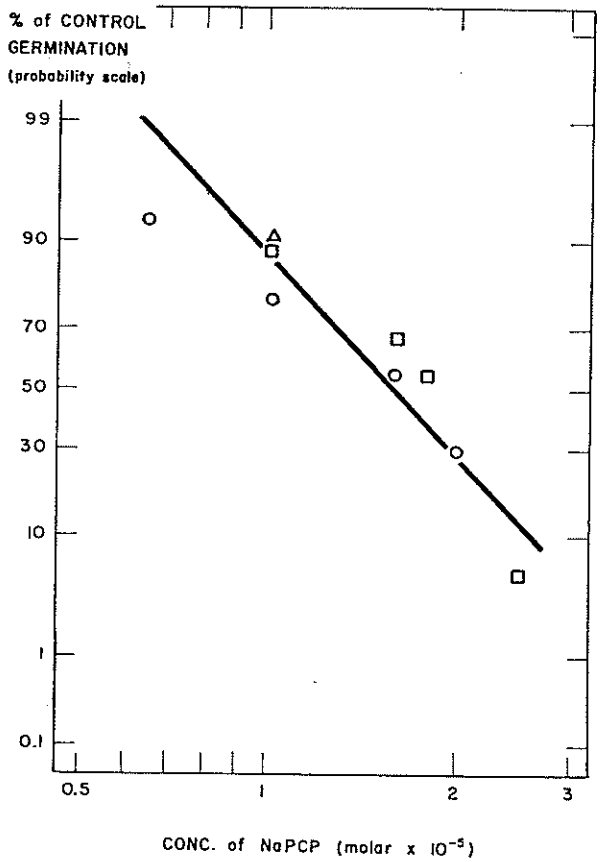

Figure 1. Inhibition of germination of spores of *Myrothecium verrucaria* in shaker flasks by sodium pentachlorophenate (NaPCP). Data from three replicate experiments. Substrate = sucrose, 0.1 per cent; yeast extract, 0.1 per cent. Incubation = 4 hr at 30 C. Spore concentration = 2.7 to 4.4 $\times$ 10^5 per ml. Age of spores = 14 days.

pentachlorophenate (NaPCP) was tested at 10^{-6} to 10^{-4} M. An average curve, fitted by eye, indicates that the ED_{50} (dosage for 50 per cent inhibition) is about 1.8×10^{-5} M NaPCP.

Data of the progress of germination with time are readily obtained by taking successive harvests from the same flasks. This is illustrated in figure 2 which shows the germination curves for four levels of copper 8-quinolinolate (Cu-8). The same data are presented in table 1 expressed as per cent of control germination. This shows the relative effect of Cu-8 with time.

The use of the method for fungicide combinations is illustrated in table 2. In this experiment zinc dimethyldithiocarbamate (ZnDDC) and 2-mercaptobenzothiazole (2-MBT) were combined factorially over their partially effective ranges.

The results show that various combinations of the two compounds may have qualitatively different effects on germination. Thus, the addition of 1×10^{-6} M ZnDDC to 1×10^{-5} M 2-MBT causes a reversal of the effect of the latter, and the addition of 1×10^{-5} M 2-MBT to 3.16×10^{-6} M ZnDDC also causes a reversal. Other combinations suggest no joint effect except that with 3.16×10^{-5} M 2-MBT and 2×10^{-6} M ZnDDC there is a greatly enhanced inhibition of germination which suggests a synergistic effect.

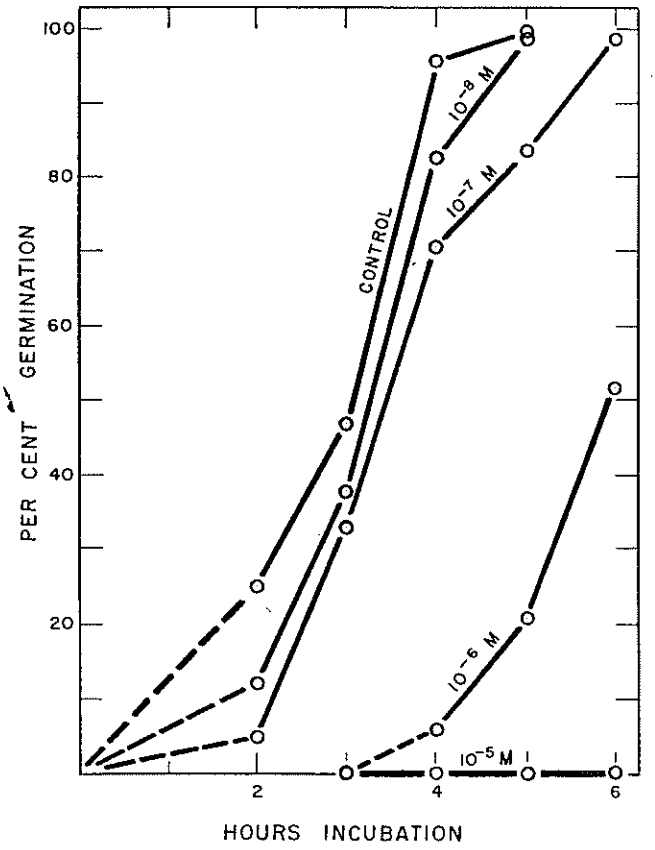

Figure 2. Inhibition of germination of spores of *Myrothecium verrucaria* in the presence of copper 8-quinolinolate. Substrate = sucrose, 0.1 per cent; yeast extract, 0.1 per cent. Harvest at 2, 3, 4, 5, and 6 hr. Spore concentration = 4.8×10^5 per ml. Age of spores = 8 days.

Discussion

The rather good agreement of the results of the three replicate experiments in figure 1 compares favorably with results obtained by more conventional methods (Horsfall, 1956). The speed and ease with which they were obtained, the elimination of aseptic techniques, and better control of conditions such as pH, aeration, and nutrition afforded by the shaker flasks recommend the method for routine or detailed studies of fungicides.

The choice of 4 hr incubation as a standard was made from studies as illustrated in figure 2. At 4 hr the control flasks show essentially 100 per cent germination, and the experimental flasks show the greatest differentiation.

Prolonged incubation or too dense a suspension is likely to cause some clumping as the sporelings elongate, and this may interfere with accurate counting. Conversely, a short incubation militates against adaptation of the organism to the fungicide.

Other organisms have been successfully used including *Memnoniella echinata*, *Alternaria tenuis*, and several

TABLE 1

*Germination of spores of Myrothecium verrucaria in the presence of copper 8-quinolinolate (Cu-8)**

Conc of Cu-8	Per Cent of Control Germination† at Hr:				
	2	3	4	5	6
M					
10^{-8}	48	80	80	99	
10^{-7}	20	70	68	84	99
10^{-6}	0	0	6	21	52
10^{-5}	0	0	0	0	0

* Conditions as for figure 2.

† Germination of controls without Cu-8 taken as 100 per cent.

TABLE 2

*Germination of spores of Myrothecium verrucaria in the presence of mixtures of zinc dimethyldithiocarbamate (ZnDDC) and 2-mercaptobenzothiazole (2-MBT)**

Conc of 2-MBT	Per Cent Germination per Conc of ZnDDC (M):			
	0 (control)	1×10^{-6}	2×10^{-6}	3.16×10^{-6}
M				
0 (control)	100	100	88	0
1×10^{-5}	80	100†	61	36†
3.16×10^{-5}	69	70	21‡	7
1×10^{-4}	13	11	0	0

* Substrate = sucrose, 0.1 per cent; yeast extract, 0.1 per cent. Incubation = 4 hr at 30 C. Spore concentration = 4.2×10^{-5} per ml. Age of spores = 28 days.

† Reversal.

‡ Synergism.

species of *Aspergillus* and *Penicillium*. *Saccharomyces cerevisiae* presented some difficulty in determining which cells had budded or divided. In general, the organisms best adapted to the technique are those that produce large single-celled spores borne profusely in a moist condition and which germinate readily on a simple medium.

SUMMARY

A rapid, simple method is proposed for evaluating fungicides by the inhibition of spore germination in shaker flasks. This method is completed in a few hours incubation, does not require asepsis, is reproducible, and is suitable for a variety of applications.

REFERENCES

DAVIES, O. L., DUCKWORTH, R. B., AND HARRIS, G. C. M. 1948 A method for estimating percentage germination of fungal spores. Nature, **161,** 642.

FOSTER, J. W. 1949 *Chemical activities of fungi.* Academic Press, Inc., New York, New York.

HORSFALL, J. G. 1956 *Principles of fungicidal action.* Chronica Botanica Co., Waltham, Massachusetts.

KLUYVER, A. J. AND PERQUIN, L. H. C. 1933 Zur Methodik der Schimmelstoffwechseluntersuchungen. Biochem. Z., **266,** 68–81.

MANDELS, G. R. AND NORTON, ANNA B. 1948 Studies on the physiology of spores of the cellulolytic fungus *Myrothecium verrucaria*. Quartermaster General Laboratories Research Report, Microbiology Series No. **11.**

MANDELS, G. R. AND DARBY, R. T. 1953 A rapid cell volume assay for fungitoxicity using fungus spores. J. Bacteriol., **65,** 16–26.